

Neurotoxicity and Behavioral Effects of Thiram in Rats

by Cheng-Chun Lee* and Paul J. Peters*

Eight of 24 female rats fed 66.9 mg/kg-day of thiram developed neurotoxicity. The neurotoxic effects were characterized by ataxia and paralysis of the hind legs. There were demyelination, degeneration of the axis cylinders, and presence of macrophages in the nerve bundle of the sciatic nerve. Degeneration in the ventral horn of the lower lumbar region of the spinal cord was evidenced by chromatolysis of motoneurons, pyknosis, and satellitosis. During a second experiment, 4 of 24 females fed 65.8 mg/kg-day also developed ataxia and paralysis. An additional 9 females showed claspings of the hind feet when picked up by the tail. Nerve conduction could not be measured for one severely ataxic rat and the electromyogram indicated a loss of motor unit function. Histopathology of this rat, along with the others, suggests the peripheral nerve as the primary site of the lesion. Thiram also caused behavioral changes in apparently normal rats. The walking pattern of the hind legs was altered with decreases in stride width and the angle between contralateral steps. These rats required significantly more shock-motivations and cleared a lower height in a jump/climb ability test. An open-field study indicated that thiram caused hyperactivity in the nonataxic rats of both sexes. Three of 24 rats fed 95.8 mg/kg-day of ferbam also developed ataxia or paralysis.

Introduction

The discovery of the dithiocarbamates early in the history of organosulfur chemistry led to important uses in the rubber industry. Their profound effects on biological systems due to their metal-combining capacity and their ability to interact with sulfhydryl-containing compounds have practical applications in the fields of medicine and agriculture. Thiram (tetramethylthiram disulfide) was described for the treatment of scabies (1) and is an ingredient of commonly used soap and lotions on the market today. A closely related compound, thiuram (tetraethylthiuram disulfide, better known as antabuse) was introduced for the correction of chronic alcoholism in 1948 (2). This drug has found extensive use in some European countries.

Since the first report on the insect repellent activity of some thiuram disulfides (3), reports on the insecticidal and acaricidal activities of the dithiocarbamates have been greatly multiplied. For instance, the metal salts of ethylenebisdithiocarbamic acid were reported to be effective in the control of the greenhouse whitefly (4).

The discovery of fungicidal action of various dithiocarbamates and thiuram sulfides in the United States (5) and in Great Britain (6) led to the recognition of this class of compounds as potential agricultural fungicides. The fungicidal activity of the ethylenebisdithiocarbamates was discovered in 1943 (7, 8). Disodium ethylenebisdithiocarbamate, the parent compound, is highly water-soluble and unstable in air. The stabilizing effect of a zinc sulfate-lime mixture (9) was attributed to zinc salt formation (10).

Of the many dithiocarbamates synthesized and studied, mainly by the agricultural chemical industry, a few have been widely used as fungicides. Of those derived from dialkylamines, only the iron and zinc salts of dimethyldithiocarbamic acid (ferbam and ziram) and tetramethylthiuram disulfide (thiram) have been widely accepted. Of the ethylenebisdithiocarbamates, only the disodium, zinc and manganese salts (nabam, zineb, and maneb) are extensively used.

As part of a long-range safety evaluation, we have studied the disposition and metabolism (11), developmental toxicity (12), acute, subacute and chronic toxicity of ferbam and thiram (13), and the cytotoxicity of dithiocarbamates (14). After long-term feeding of thiram or ferbam, some rats developed neural dysfunctions. This communication describes these ef-

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fects and neuropathology in rats fed thiram. In addition, neurofunction of the ataxic rats and behavioral changes in apparently normal rats fed thiram were studied.

Experimental Animals and Incidence of Neurotoxicity

For thiram and ferbam, 96 healthy males and 96 healthy females of the Charles River CD strain (Wilmington, Mass.) were randomly divided into four groups of 24 rats each. The rats were housed, two per cage, in plastic cages with filter tops in rodent quarters maintained at $74 \pm 2^\circ\text{F}$ and $50 \pm 10\%$ relative humidity on a 12-hr light cycle.

Standard rodent feed was analyzed and found to contain insignificant amounts of chlorinated hydrocarbons and PCB. Diets were prepared containing 0% (control), 0.01%, 0.04%, and 0.1% of the test compounds. As the rats grew, feed intake as well as compound intake/rat per day decreased. Therefore, the percentage of compound in the diet was periodically increased to give a relatively constant consumption on basis of body weight. Over the entire feeding period of 80 weeks, active thiram intake oscillated around 5.3, 20.4, and 52.0 mg/kg-day for the males; and 6.1, 25.5, and 66.9 mg/kg-day for the females. Active ferbam intake oscillated around 7.7, 31.9, and 79.7 mg/kg-day for the males; and 9.3, 36.6, and 95.8 mg/kg-day for the females.

A second experiment with thiram utilized two groups of 24 female rats which were housed four per hanging cage. One group received powdered feed only, and the other received feed plus 0.1% thiram. As for the first study, the thiram concentration was altered and the active thiram intake averaged 65.8 mg/kg-day for 36 weeks.

Eight female rats fed the high level of thiram during the first experiment developed hind limb ataxia or paralysis. This was first observed in one rat during the 5th month, two more occurred during the 13th month, one during the 17th month, two during the 18th month, and two during the 19th month when treatment was discontinued. The ataxia syndrome consisted of an unusual gait with dragging of the hind feet and tail. Figure 1 shows a typical ataxic rat and Figure 2 a partially ataxic rat. The condition of these rats worsened and their

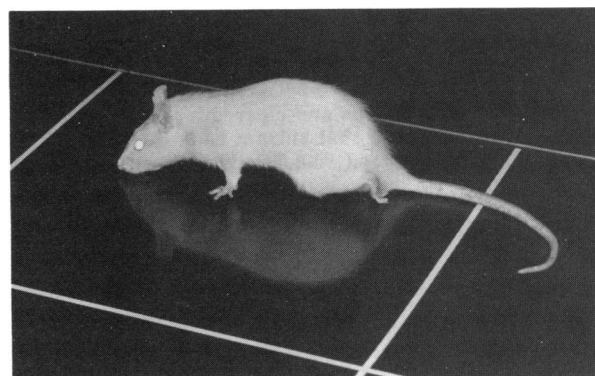
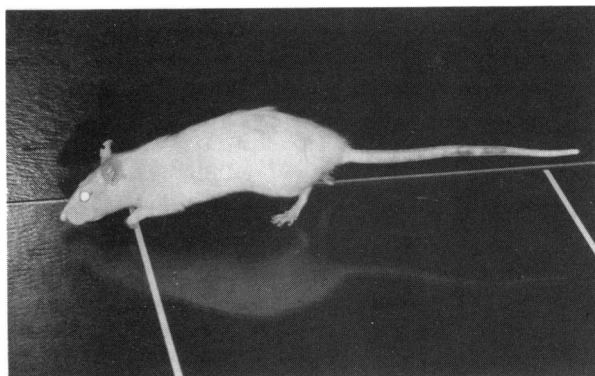


FIGURE 1. Female rats fed high level thiram: (top) nonataxic rat; (bottom) ataxic rat.

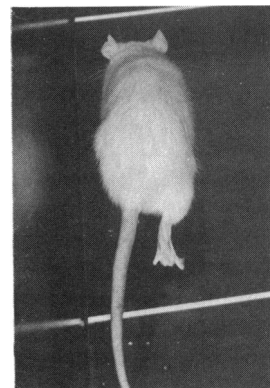
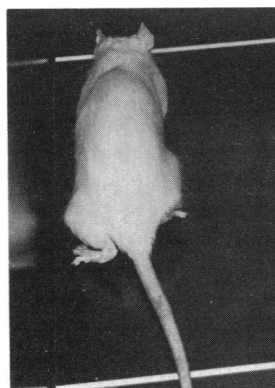


FIGURE 2. Female rats fed high level of thiram: (right) nonataxic rat; (left) partially ataxic rat.

hind legs were atrophied. These effects led to paralysis posterior to the lumbar region and some rats showed severe curvature of the thoracic spine. Near termination, these rats were extremely emaciated. Some female rats fed the high level of thiram walked by swinging their hind legs widely to the side and then back in a rearward breast stroke. This may be a prelude to ataxia. In addition, patches or wide areas of alopecia occurred over the body of some ataxic and nonataxic rats and a few rats fed the middle dose of thiram. These and other effects will be reported elsewhere (13). Three female rats fed the high level of ferbam also developed the neural effects as seen in the rats fed the high level of thiram.

It has been reported that after chronic feeding with ferbam, some rats crossed, or clasped, their hind feed when picked up by the tail (15). This was occasionally observed during the first thiram experiment and was systematically recorded during the second experiment. Of the 24 rats in the second experiment, 13 showed hind leg clasping during the 11th through 27th week. Four of these rats developed the ataxia syndrome and then paralysis during this period.

Neurofunction

Three control and three ataxic rats from the second study were lightly anesthetized with sodium pentobarbital. Maximum conduction velocities of the tibial nerve were normal for all six rats: 32, 36, and 50 m/sec for the controls; and 30, 40, and 50 m/sec for the thiram-treated rats. Spontaneous motor unit activity in the cranial tibial muscle was also normal in these rats. A typical record of one rat is shown in the top tracing of Figure 3. Three days later, the condition of this rat worsened. At this time, the nerve was unable to conduct the stimulus, and no conduction velocity recording could be made. The electromyograph was strikingly abnormal (Fig. 3, bottom tracing). Most of the motor units had ceased firing, the peaks were broader, and the baseline was uneven.

Neuropathology

An attempt was made by light microscopy to localize the lesions responsible for ataxia. Two high dose female rats showing hind limb ataxia were anesthetized and perfused through the left ventricle with physiological saline fol-

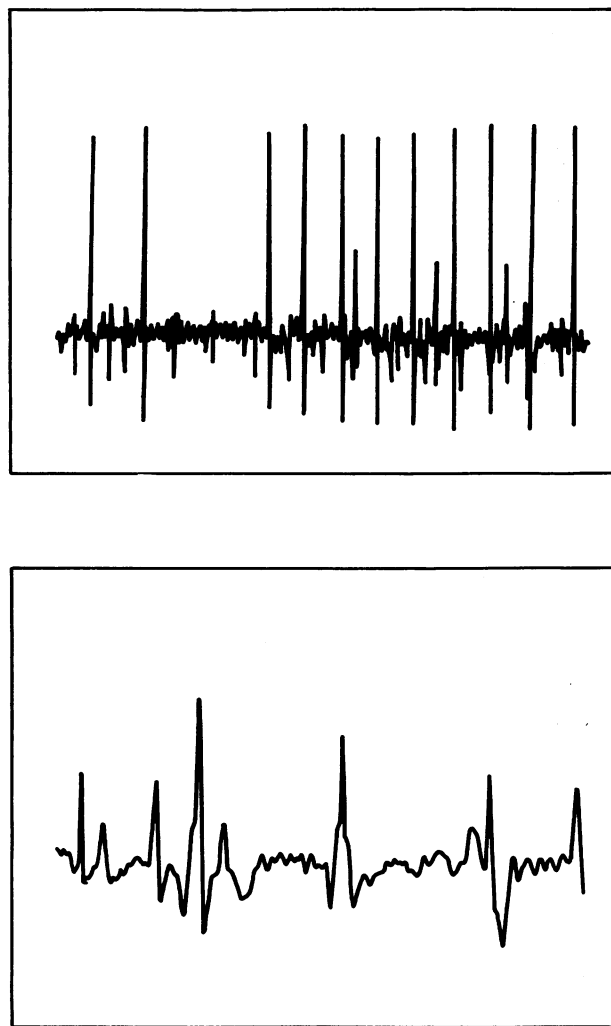


FIGURE 3. Electromyograph from cranial tibial muscle (sweep time, 200 msec): (top) ataxic rat; (bottom) same rat 3 days later.

lowed by 10% formalin. Blocks of the gastrocnemius muscle, pieces of the sciatic nerve and the spinal cord were removed for addition fixation. All tissues were embedded in paraffin and cut at 6 μ m. Longitudinal sections of the sciatic nerve were stained with Luxol Fast Blue for myelin, and by the Bodian method for axis cylinders. Cells of the spinal cord were stained by Nissl technique. Motor end plates of the muscle were stained by the Cole procedure (16).

Some demyelination was detected in the sciatic nerve of both rats (Fig. 4). Normal myelin presented a regular, small-beaded appearance whereas degenerated myelin showed large, ir-

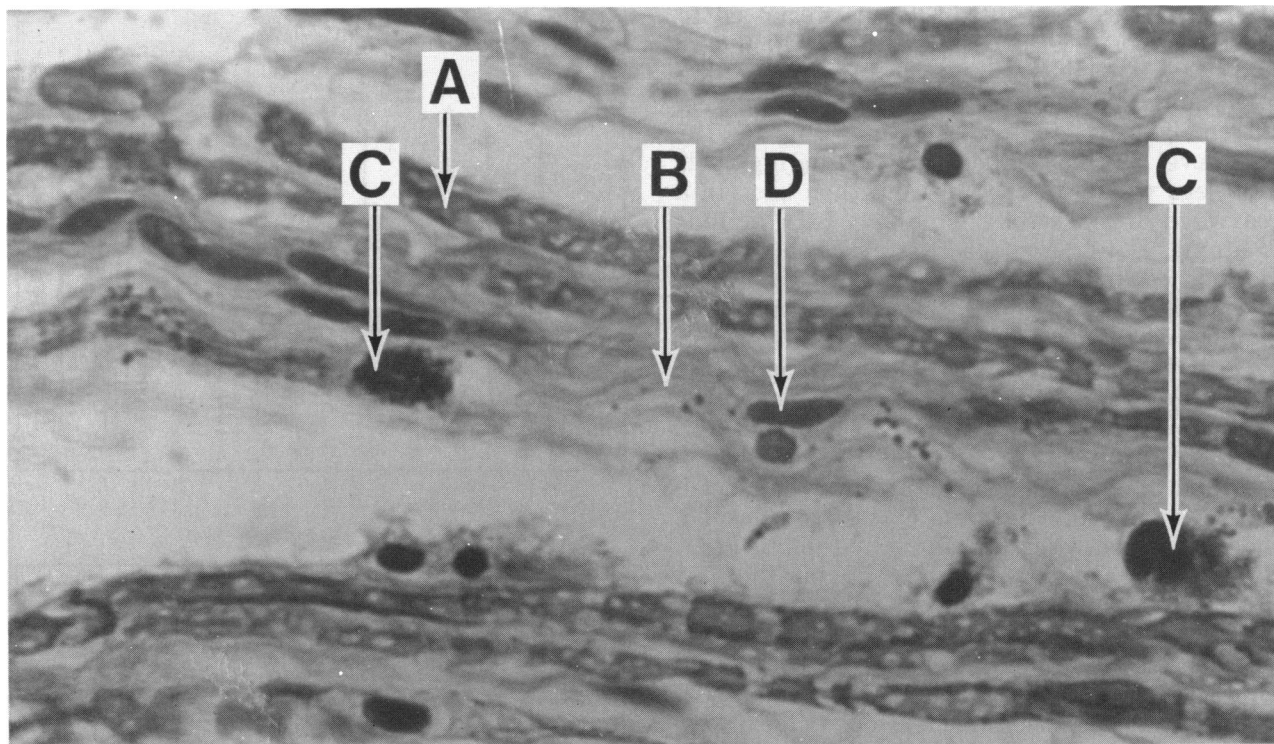


FIGURE 4. Sciatic nerve from ataxic rat: (A) normal myelin; (B) degenerating myelin; (C) macrophage; (D) Schwann cell. Luxol Fast Blue (695 \times).

regular beading and fragmentation. The presence of macrophages in the peripheral nerve bundle also indicated degeneration. The axis cylinders presented a similar degree of degeneration (Fig. 5). Sections of the lower lumbar region of the spinal cord of both rats also showed evidence of degeneration. These indications included chromatolysis of motor neurons, pyknosis in the ventral horn, and satellitosis (Fig. 6). The apparent lack of motor end plates in the gastrocnemius muscle was probably due to technical problems. It is unlikely that all the plates were degenerated.

Histopathology on the rat with the abnormal electromyograph from the second experiment showed demyelination and degeneration of the peripheral nerve, severe atrophy of the muscle (Fig. 7), and degenerative changes in the ventral horn of the lumbar cord. As seen from the first study, it would appear that the peripheral axon was the locus of the primary lesion. Muscle degeneration was probably secondary due to denervation, and the CNS effects due to retrograde degeneration.

Behavioral Effects

Although ataxia was obvious in a number of female rats fed the high level of thiram or ferbam, it was not obvious whether the apparently nonataxic rats of both sexes were affected or not. Behavioral studies were performed on rats fed various levels of thiram to look for evidence of neurotoxic effects more subtle than outright ataxia or paralysis.

Hind Leg Walking Gait

During the 79th week of feeding, the hind leg walking gait in the apparently normal females was analyzed. For this experiment, the rat's hind feet were dipped in ink and the rat was required to walk up an inclined board through a covered walkway. The board was covered with a sheet of polygraph paper and the rats were walked over it until a good record of six consecutive steps was obtained. The stride width, length, and the angle formed between consecutive steps on contralateral sides were calculated (17,18) as summarized in

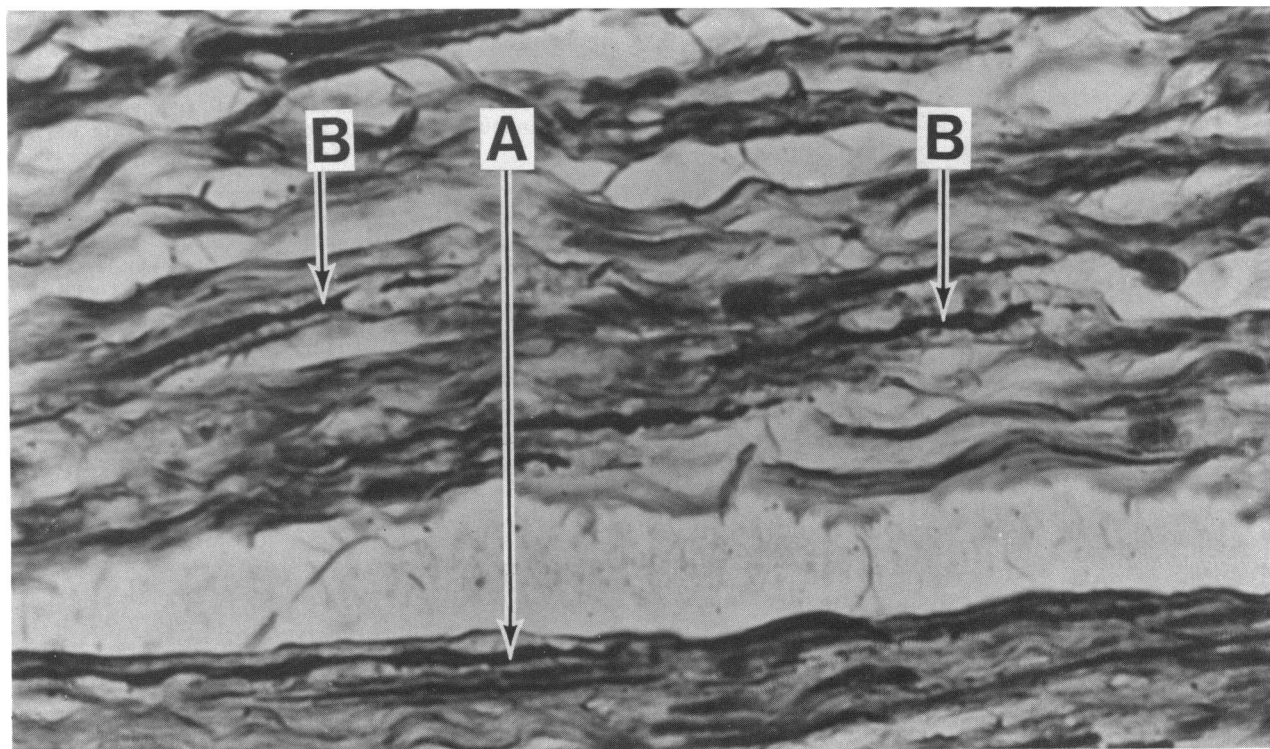


FIGURE 5. Sciatic nerve from ataxic rat: (A) normal axis cylinder; (B) degenerating axis cylinder. Bodian (695 \times).

Table 1. An analysis of variance of these data showed that there were significant dose and subject effects for each measurement ($p < 0.01$). Further analysis indicated that the stride width and the angle between contralateral steps for the rats fed the middle or the high level of thiram were significantly less than those for the control rats or rats fed the low level of thiram ($p < 0.05$).

Table 1. Means of three measures of the hind leg walking gait for female rats during the 79th week of thiram feeding.

	Number of rats	Stride length, mm ^a	Stride width, mm ^a	Angle between feet ^a
Control	14	127	44	35°
Low dose	11	122	44	36°
Mid dose	10	134	37 ^b	29° ^b
High dose	10	128	36 ^b	30° ^b
S.E.M.		± 4.3	± 0.6	$\pm 1.5^\circ$

^a Significant dose and subject effects, ANOV, $p < 0.01$.

^b Significantly different from control and low dose groups (19), $p < 0.05$.

Jump/Climb Ability Test

During the 79th week of feeding, the jump/climb test was performed to investigate the motor ability of the apparently normal female rats in a more physically demanding task. The objectives were to see how many electrical shocks would be required and how high the rats could jump/climb.

The apparatus was a two-way shuttle box, measuring $70 \times 20 \times 10$ cm ($l \times w \times h$), with the center divider and lids removed. The floor consisted of stainless steel rods. A plywood sideboard extended the height of the entire apparatus to 38 cm. The grid floor in half the chamber could be covered by plywood inserts which were 1.9 cm thick. The procedure was to use the inserts to provide a safe side in the box. In order to escape electrical shock (2 mA, 0.3 sec duration) given through the grid floor, the rat was required to jump or climb onto the safe side. When the rat reached the safe side, she was removed and stroked while one or more inserts were added. Thus,

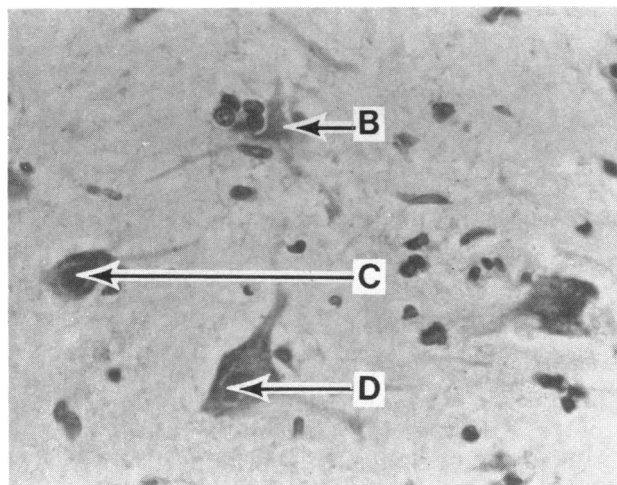
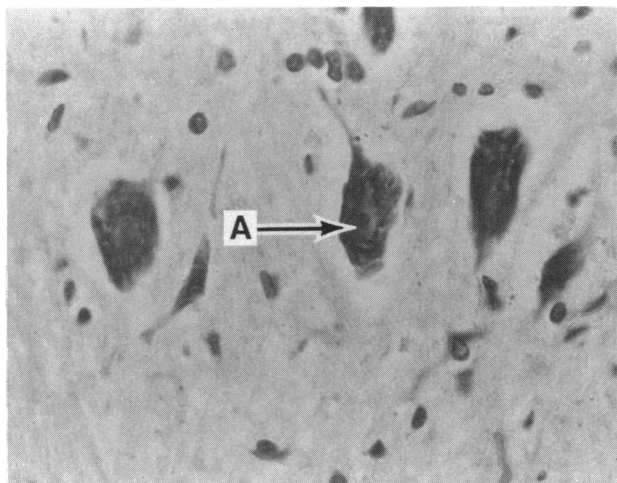


FIGURE 6. Ventral horn (lower lumbar region): (A) normal motor neuron; (B) satellitosis; (C) pyknosis; (D) chromatolysis. Nissl (284 \times).

the safe side was increased in height a minimum of 1.9 cm after each successful escape. A maximum of 20 inserts totalling 38 cm was used. The shock used as motivation was applied by the experimenter whenever the rat turned away from the safe side or otherwise made no attempt to jump out. If a rat failed repeatedly to reach the safe side, no further shock was given and the maximum height reached was recorded.

The nonataxic females fed the high level of thiram required significantly more shocks ($p < 0.01$) and cleared a lower height ($p < 0.01$)

than did the control rats and rats fed the low or middle level of thiram (Table 2). All rats

Table 2. Number of shocks received and height scaled (maximum of 38 cm) by female rats during the 79th week of thiram feeding.

	Number of rats	Shocks ^a	Height, cm
Control	14	7.1 \pm 0.9	38
Low dose	14	6.2 \pm 0.6	38
Mid dose	13	6.7 \pm 1.1	38
High dose	10	14.8 \pm 2.2 ^b	31 \pm 5 ^b

^a Mean \pm S.E.M.

^b Significantly different from the control (20), $p < 0.01$.

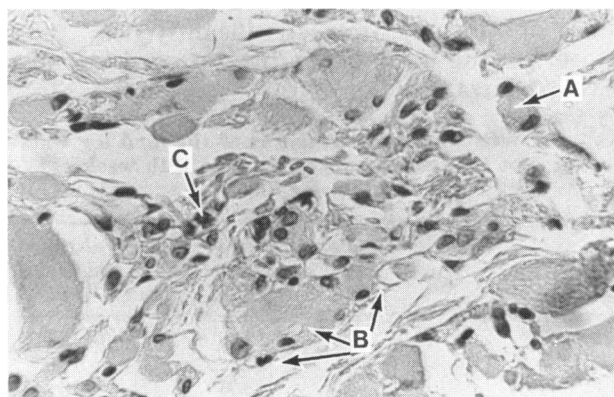
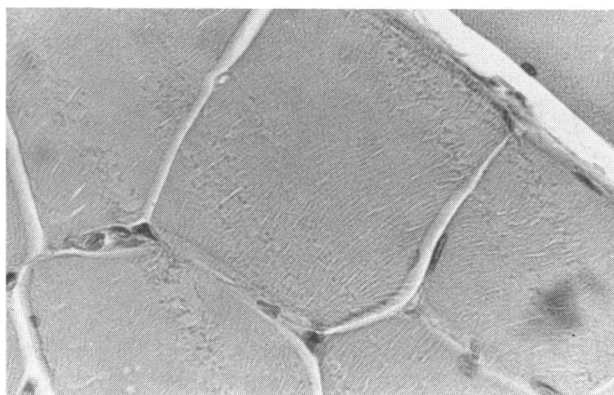


FIGURE 7. Gastrocnemius muscle; (top) normal rate; (bottom) ataxic rat, (A) degenerating muscle fibers; (B) atrophy of muscle bundles; (C) mononuclear cells; H and E (210 \times).

except those fed the high level cleared the maximum height available. The results suggest that nonataxic females had a mild form of the motor deficit which affected the hind legs. Since these females required more shock-motivations and showed inappropriate jumping responses, deficits in emotional or learning processes may also be indicated.

Open-Field Test

During the 80th to 82nd week of thiram feeding, males and females were tested in an open field. The apparatus was constructed of plywood and consisted of a 120 × 120 cm floor with 30.5 cm sides. The entire apparatus was painted flat black. The floor was marked off into 30.5 cm squares and was covered with clear Plexiglas. Each rat was placed in the center of the floor and observed for 5 min. The experiment was conducted in dim light with rats tested in a counter-balanced order.

Observations recorded were the number of squares entered, the incidence of rearing on the hind legs, and the number of fecal boluses. An analysis of variance showed a significant dose effect on the number of squares entered (Table 3). There was a marginal sex with dose

Table 3. Averaged open-field data for male and female rats fed thiram for 80–82 weeks.

	Number of rats	Squares ^a	Rearing ^b	Boluses
Males				
Control	14	39	16	1
Low dose	12	37	16	1
Mid dose	14	59	19	2
High dose	14	51	21	1
Females				
Control	14	27	8	0.5
Low dose	14	35	14	1
Mid dose	14	39	14	1
High dose	14	69	12	3
S.E.M.		± 6.9	± 2.8	± 0.4

^a Significant dose effect, ANOV, $p < 0.01$, and a marginal sex × dose interaction, $p < 0.10$.

^b Significant sex difference only, ANOV, $p < 0.05$

interaction due to high activity levels in both the mid and high dose males, whereas it was the high dose group alone that accounted for most of the hyperactivity of the females. Thus the results of this experiment indicated that

thiram led to hyperactivity in both male and female rats, even in the absence of obvious effects on locomotor control. Among the females fed the high level of thiram were three ataxic rats and two rats with obviously abnormal walking patterns involving the hind legs. It is interesting to note that ataxia did not prevent these rats from showing hyperactivity. These five abnormal rats entered 28 to 173 squares, averaging 95 squares, which accounted for 49% of the total squares entered by the 14 females tested.

Discussion

Neurotoxicity occurred only in female rats fed the high level of thiram or ferbam. These neurotoxic effects are not believed to be sex-dependent. The thiram or ferbam concentration in the feed was the same for the males and the females. Due to difference in body weight and in feed consumption, the thiram or ferbam intake based on body weight was higher for the females than the males. Accordingly, the female rats stopped gaining weight after 4 to 8 months of feeding and lost weight (13) prior to the development of neurotoxicity. On the other hand, the male rats continued to gain weight throughout the study, although their weight gain was considerably less than that of the controls.

Only 8 of 24 females fed the high level of thiram in the first experiment, 4 of 24 treated females in the second experiment, and 3 of 24 females fed the high level of ferbam developed ataxia or paralysis. It is highly probable that these symptoms of neurotoxicity would occur in more rats if the experimental period was extended or if the compound intake was increased.

Thiram is more neurotoxic than ferbam. For thiram, lower intake was required to produce these effects, more rats were affected, and these effects occurred earlier. We reported that the cytotoxicity of thiram and the metal-containing dithiocarbamate fungicides indicated a common mechanism of toxic effect (14). Results from these studies suggest that ferbam converts to the dimethyldithiocarbamate moiety in the body and then it or its metabolites lead to neurotoxicity. One metabolite, carbon disulfide, was detected in the expired air of rats treated orally with ferbam (11). Carbon disulfide has been reported to cause neurotoxicity, including

demyelination, in experimental animals and man (21). The more stable ferbam and the metabolic conversion might explain the lesser toxicity of ferbam as compared with the bisdimethyldithiocarbamate, thiram.

Neurotoxic effects have been established for a number of dithiocarbamates including ferbam, ziram (zinc dimethyldithiocarbamate) and thiram in rats (15), thiram in chickens (22), antabuse (tetraethylthiuram disulfide) in rats (23) and in man (24-26), ethyl namate (sodium diethyldithiocarbamate) in hens (27) and in rabbits (28), and tecoram (an oxidation product of disodium ethylenebisdithiocarbamate and ethyl namate with ammonium persulfate) in chick embryos (29). Two carbamate pesticides, carbaryl (1-naphthyl-*N*-methylcarbamate) and aprocarb (2-isopropoxyphenyl-*N*-methylcarbamate), were also reported to be neurotoxic in rats and swine (30-32). After chronic administration of ferbam and ziram, a hind leg "grasping" reaction was observed in rats (15). However, the more severe ataxia syndrome including paralysis and neuropathology, were not found. Both the male and female rats were fed 0.25% of ferbam and ziram. Their growth was retarded, but none of the rats fed ferbam survived the two-year period. In addition, both male and female rats fed 0.125% thiram developed the hind leg "grasping" reaction. The daily intake of ferbam, ziram or thiram was not measured and the purity of these compounds was not given in their study.

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REFERENCES

- Gordon, R. M., and Seaton, D. R. Observations on the treatment of scabies. *Brit. Med. J.* 1942 (4248): 685 (1942).
- Hald, J., and Jacobsen, E. A drug sensitizing the organism to ethyl alcohol. *Lancet* 255: 1001 (1948).
- Guy, H. G. Thiuram sulfides as repellents to leaf-feeding insects. *J. Econ. Entomol.* 29: 467 (1936).
- McCullen, R. D. Insecticidal action of ethylenebisdithiocarbamates. *Nature* 184: 1338 (1959).
- Tisdale, W. H., and Williams, I. (to E. I. du Pont de Nemours & Company). Disinfectant and fungicide. U.S. Pat. 1,972,961 (September 11, 1934).
- Montgomery, H. B. S., Moore, M. H., and Shaw, H. Field trials in 1935 of the fungicidal and phytocidal properties of certain new chemical preparations. *Ann. Rept. East Malling Research Sta. Kent.* 1935: 198 (1936).
- Hester, W. F. (to Rohm and Haas Company). Fungicidal composition. U.S. Pat. 2,317,765 (April 27, 1943).
- Diamond, A. E., Heuberger, J. W., and Horsfall, J. G. A water-soluble protectant fungicide with tenacity. *Phytopathology* 33:1095 (1943).
- Heuberger, J. W., and Manns, T. F. Effect of zinc sulfate-lime on the protective value of organic and copper fungicides against early blight of potato. *Phytopathology* 33: 1113 (1943).
- Barratt, R. W., and Horsfall, J. G. Fungicidal action of metallic alkylbisdithiocarbamates. *Conn. Univ. Storrs Agr. Expt. Sta. Bull.* 1947: 508.
- Hodgson, J. R., et al. Metabolism and disposition of ferbam in the rat. *Toxicol. Appl. Pharmacol.* 33: 505, (1975).
- Short, R. D., Jr., et al. Developmental toxicity of ferric dimethyldithiocarbamate and bis(dimethylthiocarbamoyl) disulfide in rats and mice. *Toxicol. Appl. Pharmacol.* 35: 33 (1976).
- Lee, C. C., Russell, J. Q., and Minor, J. L. Acute, subacute and chronic toxicities on dithiocarbamate fungicides in rodents. In preparation.
- Hodgson, J. R., and Lee, C. C. Cytotoxicity studies on dithiocarbamate fungicides. *Toxicol. Appl. Pharmacol.*, in press.
- Hoge, H. C., et al. Chronic oral toxicity of ferric dimethyldithiocarbamate (ferbam) and zinc dimethyldithiocarbamate (Ziram). *J. Pharmacol. Expt. Therap.* 118: 174 (1956).
- Thompson, J. R., and Matzke, H. A. Effects of ischemia on the hind limb of the rat. *Am. J. Phys. Med.* 54: 113 (1975).
- Rushton, R., Steinberg, H., and Tinson, C. Effects of a single experience on subsequent reactions to drugs. *Brit. J. Pharmacol.* 20: 99 (1963).
- Mullenix, P., Norton, S., and Culver, B. Locomotor damage in rats after x-irradiation *in utero*. *Expl. Neurol.* 48: 310 (1975).
- Duncan, D. B. Multiple range and multiple *F* tests. *Biometrics* 11:1 (1955).
- Dunnett, C. W. A multiple comparisons procedure for comparing several treatments with a control. *J. Amer. Statist. Assoc.* 50: 1096 (1955).
- Gordy, S. T., and Trumper, M. Carbon disulfide poisoning. *J. Amer. Med. Assoc.* 110: 1543 (1938).
- Waibel, P. E., et al. Toxicity of tetramethylthiuram disulfide for chicken, poult and goslings. *Poultry Sci.* 36: 697 (1957).
- Fitzhugh, O. G., Winter, W. J., and Nelson, A. A. Some observations on the chronic toxicity of Antabuse (tetraethylthiuram disulfide). *Fed. Proc.* 11: 345 (1952).
- Charatan, F. B. Peripheral neuritis following tetraethylthiuram disulfide treatment. *Brit. Med. J.* 2: 380 (1953).

25. Barry, W. K. Peripheral neuritis following tetraethylthiuram-disulphide treatment. *Brit. Med. J.* 2: 937 (1953).
26. Bradley, W. G., and Hewer, R. L. Peripheral Neuropathy Due to Disulfiram. *Brit. Med. J.* 2: 449 (1966).
27. Howell, J. M., and Edington, N. The neurotoxicity of sodium diethyldithiocarbamate in the hen. *J. Neuropathol. Expt. Neurol.* 27: 464 (1968).
28. Edington, H., and Howell, J. M. The neurotoxicity of sodium diethyldithiocarbamate in the rabbit. *Acta Neuropathol.* 12: 339 (1969).
29. Van Steenis, G., and Van Logten, M. J. Neurotoxic effect of the dithiocarbamate tecoram on the chick embryo. *Toxicol. Appl. Pharmacol.* 19: 675 (1971).
30. Gaines, T. B. Acute toxicity of pesticides. *Toxicol. Appl. Pharmacol.* 14: 515 (1969).
31. Smalley, H. E., et al. The effect of chronic carbaryl administration on the neuromuscular system of swine. *Toxicol. Appl. Pharmacol.* 14: 409 (1969).
32. Desi, I., et al. Neurotoxicologic studies of two carbamate pesticides in subacute animal experiments. *Toxicol. Appl. Pharmacol.* 27: 465 (1974).